Remarks

The applicants and the undersigned would like to thank Examiners Kosson and Hutson for the courtesy extended to the undersigned during the telephonic interview on October 19, 2006. The undersigned is generally in agreement that the Interview Summary mailed on October 25 summarizes the substance of the interview.

Claims 1-15 and 21-31 were the subject of the final office action dated July 5, 2006. Claims 3, 9, 11, 13, 15, and 26-31 were withdrawn from consideration as being drawn to non-elected subject matter. By this response, claims 3, 6, 8, 9-11, 13, 15, 26-29, and 31 are canceled without prejudice, and claims 32-33 are added. Thus, claims 1, 2, 4, 5, 7, 12, 14, 21-25, 30, and 32-33 are before the examiner for further consideration.

Issues raised in the office action will be addressed first. Issues raised during the interview and in the Interview Summary will be then be addressed, below.

Various claims are amended and canceled without prejudice to remove reference to non-elected sequences and the like.

The nomenclature in claim 12 is corrected per the suggestion in the office action.

Regarding written descriptions of fragments and the like, new claims 32 and 33 include a specific reference to fragments of the A protein. Paragraph 21 of the specification discusses protease processing of the A proteins. Examples 16 and 27 of U.S. Patent No. 6,528,484 also show that protease-activated A proteins retain insecticidal activity. Various sections of the specification thus describe the production and use of such fragments according to the subject invention (with mixed/heterologous potentiators).

Regarding variants and hybridization conditions, a wide range of B and C potentiators were shown to be useful for potentiating the activity of the elected Protein A. These potentiators include TcdB1, TcdB2, TcaC, XptC1, XptB1, and PptB1, and TccC1, TccC2, TccC3, TccC5, XptB1, XptC1, and PptC1. Thus, a range of variants of the elected sequences have been shown to work, and would be expected to work, according to the subject invention.

The Written Description Guidelines show that the construction and use of such variants is known in the art. (The subject invention relates to novel combinations of diverse components, which were not heretofore suspected or suggested to be active in combination.) Furthermore, 0.1X SSC and 55°C are highly stringent conditions. As "highly stringent" conditions, the

Written Description Guidelines use 6X SSC and 65°C. Although that temperature is slightly higher, the salt concentration is much higher (and thus, less stringent).

The comments above should also address the enablement issues raised in the office action.

Regarding the indefiniteness issue raised on page 7 of the office action, specifying the temperature and salt concentration are believed to be the main components of hybridization conditions. Selecting an appropriate number of washes and duration of each is within the skill in the art and is discussed in the specification.

The prior art rejection is discussed in more detail below with reference to the issues raised during the interview.

Regarding the Interview Summary, claim 1 is amended to specify that Proteins A, B, and C are complex-forming proteins, that Protein A has activity against an insect, and that Proteins B and C potentiate the activity of Protein A.

The industry-standard nomenclature, "toxin complex" proteins, indicates that these components form a complex. *See, e.g.,* Waterfield *et al.* (2001), "The tc genes of Photorhabdus: a growing family," *Trends in Microbiology* 9:185-91. Complex formation is discussed in more detail below.

Paragraph 158 of the specification states, "By 'functional activity' (or 'active against') it is meant herein that the protein toxins function as orally active insect control agents (alone or in combination with other proteins), that the proteins have a toxic effect (alone or in combination with other proteins), or are able to disrupt or deter insect growth and/or feeding which may or may not cause death of the insect. [underlining added]"

The specified activity is somewhat akin to "food poisoning." While "poison" implies something that can kill, most people do not typically die from food poisoning. They typically become very sick (and lose their appetites). If insects are rendered sufficiently "sick" by the combined toxin activity of the subject proteins, they will stop eating the resistant or treated plants. Insects can also be killed outright by the subject proteins. In either case, the desired result is protection of plants from damage by insects.

Paragraphs 31-32 of the specification state:

> While the exact molecular interactions of the TC proteins with each other, and their mechanism(s) of action, are not currently understood, it is known, for example, that the Tca toxin complex of *Photorhabdus* is toxic to *Manduca sexta.* In addition, some TC proteins are known to have "stand alone" insecticidal activity, while other TC proteins are known to potentiate or enhance the activity of the stand-alone toxins. It is known that the TcdA protein is active, alone, against *Manduca sexta,* but that TcdB and TccC, together, can be used to enhance the activity of TcdA. Waterfield, N. *et al., Appl. Environ. Microbiol.* 2001, 67:5017-5024. TcbA (there is only one Tcb protein) is another stand-alone toxin from *Photorhabdus*. The activity of this toxin (TcbA) can also be enhanced by TcdB together with TccC-like proteins.
>
> U.S. Patent Application 20020078478 provides nucleotide sequences for two potentiator genes, tcdB2 and tccC2, from the tcd genomic region of *Photorhabdus luminescens* W-14. It is shown therein that coexpression of tcdB and tccC1 with tcdA in heterologous hosts results in enhanced levels of oral insect toxicity compared to that obtained when tcdA is expressed alone in such heterologous hosts. Coexpression of tcdB and tccC1 with tcdA or tcbA provide enhanced oral insect activity.

Thus, paragraph 31 and the patent application cited in paragraph 32 show that the "A+B+C" / stand-alone toxin (Protein A) + BC potentiators was known in the art. (Again, the subject invention relates to novel combinations of diverse components thereof, which were not heretofore suspected or suggested to be active in combination.)

Example 3 of the subject specification, including Tables 8 and 11-13, provide some exemplary results. (There are many other Examples, and many, many other Tables presenting similar results). Table 8 shows that A, A+B, and A+C have little or no activity, while A+B+C has very high activity. Table 11 shows that B+C is essentially inactive while A+B+C is very active. Table 12 shows that a Toxin A has some activity, and that B, C, A+C, A+B, and B+C had little or no activity, while A+B+C was very active. Table 13 shows that B+C had no activity, A had some activity, and A+B+C had very good activity.

Based on the above, it is clear that the "A" proteins are the stand-alone toxins that are responsible for specificity, and that the BC proteins potentiate that activity. It is not the case where A, B, and C are each toxic. Thus, the situation at hand is not like taking acetaminophen, ibuprofen, and naproxen sodium for aches and pains. The subject ABC proteins form a complex, interact, and work together, with A being the main toxin.

Paragraph 296 of the subject specification states, "It was an unexpected revelation that toxin complex Class A, B, and C components from strains noted for either coleopteran (*Photorhabdus luminescens* strain W-14) or lepidopteran activity (*Xenorhabdus nematophilus* strain Xwi) may be functionally mixed and matched. Additionally surprising was the discovery of the degree of divergence possible for individual A, B or C proteins." The surprising nature of the subject invention is especially evident in light of the overall sequence divergence between *Xenorhabdus* and *Photorhabdus* potentiators and their targets – the A proteins.

Incidentally, yet another surprising aspect of the subject invention is that heterologous combinations of B+C are shown to be some of the most preferred potentiators – even better than wild-type BC combinations (with heterologous A proteins).

Paragraphs 127-128 of the subject specification state:

> Although the complex of (TcbA or TcdA) + (TcaC + TccC) might appear to be a similar arrangement as the complex of (XptA1 or XptA2) + (XptC2 + XptB1), each *Photorhabdus* component shares only about 40% (approximately) sequence identity with the "corresponding" *Xenorhabdus* component. The unique TC proteins from *Paenibacillus* also share only about 40% sequence identity with "corresponding" *Photorhabdus* and *Xenorhabdus* TC proteins (those proteins and that discovery are the subject of co-pending U.S. application serial no. 60/392,633, Bintrim *et al.*, filed June 28, 2002).
>
> It is in this context that it was discovered, as described herein, that *Xenorhabdus* TC proteins could be used to enhance the activity of *Photorhabdus* TC proteins and *vice versa*. *Paenibacillus* TC proteins are also surprisingly demonstrated herein to potentiate the activity of *Xenorhabdus* (and *Photorhabdus*) TC toxins. This was not previously proposed or demonstrated, and was very surprising especially in light of the notable differences between *Xenorhabdus*, *Photorhabdus*, and *Paenibacillus* TC proteins. There was certainly no expectation that divergent proteins from these divergent organisms would be compatible with each other.

Complex formation and possible mechanisms of action are discussed in more detail throughout the specification and in the Background section. A good, contemporaneous review of related findings is reported in, for example, Sergeant *et al.* (2003), *Applied and Environmental Microbiology*, pp3344-3349, "Interactions of Insecticidal Toxin Gene Products from *Xenorhabdus nematophilus* PMFI296." A copy is enclosed.

As stated in the abstract section of that reference, xptA1, xptB1, and xptC1 were needed for activity against *P. rapae* and *brassicae*, and xptA2, xptB1, and xptC1 were needed for activity against *H. virescens*. The first full paragraph in the second column on page 3348 indicates that specificity or "spectrums of activity" is due to the "A" protein (as XptA1 and XptA2 had activities against different pests), but that the B and C proteins are responsible for increasing the activity of the A protein.

The abstract of Sergeant *et al.* concludes by stating, "Therefore, the two three gene product combinations interact with each other to produce good insecticidal activity."

The sentences bridging the columns on page 3347 state, "Thus, interaction between the xptA1 and xptB-1-xptC1 genes was essential for activity...both genes do need to be expressed by the same cell, as shown by the lack of insecticidal activity when xptB1 and xptC1 were expressed in separate cells." The last full paragraph on page 3347 also states, "These results supported the earlier findings that the xptB1 and xptC1 genes need to be in the same E. coli cell in order to be able to complement xptA1. Similarly, both genes have to be expressed within the same cell in *Xenorhabdus* strains."

It is interesting to note that Sergeant *et al.* found that the A, B, and C components had to interact or associate within the same cell.

The first paragraph of the DISCUSSION section states that, "...for full insecticidal activity...interactions between the products of three xpt genes...are required."

The first full paragraph on page 3349 discusses the formation of observable structures comprising tcdA (an "A" protein) and tcdB (a "B" protein), with the "C" protein (tccC) affecting their activity. The end of that column discusses some differences between *Xenorhabdus* and *Photorhabdus* genes in the respective genomes. The last paragraph of this article is also a good summary of those findings.

Based on all of the above, it is clear that the A, B, and C proteins need to interact with each other for toxicity. What is also noteworthy is that while this reference discusses both *Xenorhabdus* and *Photorhabdus* toxins and potentiators together, no suggestion is made therein to combine *Xenorhabdus* and *Photorhabdus* components.

In light of all the foregoing, the withdrawal of the obviousness rejection, and all the other rejections, is respectfully requested.

Still further evidence of the interactions between the subject A, B, and C proteins can be found in US-2006-0168683-A1. That patent application relates to advantageously fused ABC and BC proteins, but similar binding studies could be conducted for non-fused A, B, and C proteins. *See, e.g.,* Example 6 ("...the TcdB2/TccC3 V1 fusion protein bound XptA2 faster than the non fused TcdB2+TccC3 complex. Once bound, neither preparation dissociated readily from XptA2."); Example 15 ("All four soluble lysates containing the TcdB2/TccC3 fusions protein bound to the immobilized XptA2 strongly"); and Example 16 ("The association rate of the 8920 fusion protein (ka = 1.03×10^6) was at least 20-fold greater than the association rate of TcdB2+TccC3 (ka = 4.49×10^4). Once bound, neither protein dissociated readily from XptA2.").

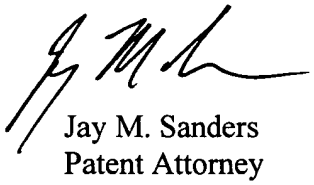
An expert declaration can be produced if a more detailed explanation of any of the foregoing would be helpful.

The applicants believe that this application is in condition for allowance, and such action is earnestly solicited.

The Assistant Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 and 1.17 as required by this paper to Deposit Account 19-0065.

The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Attachments: Request for Continued Examination (RCE) Transmittal
Sergeant *et al.* 2003 reference
Amended claims (without markings)

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Interactions of Insecticidal Toxin Gene Products from *Xenorhabdus nematophilus* PMFI296

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Four genes on a genomic fragment from *Xenorhabdus nematophilus* PMFI296 were shown to be involved in insecticidal activity towards three commercially important insect species. Each gene was expressed individually and in combinations in *Escherichia coli*, and the insecticidal activity of the lysates was determined. The combined four genes (*xptA1*, *xptA2*, *xptB1*, and *xptC1*), in *E. coli*, showed activity towards *Pieris brassicae*, *Pieris rapae*, and *Heliothis virescens*. The genes *xptA1*, *xptB1*, and *xptC1* were involved in expressing activity towards *P. rapae* and *P. brassicae*, while the genes *xptA2*, *xptB1*, and *xptC1* were needed for activity towards *H. virescens*. When each of these three genes was expressed individually in *E. coli* and the cell lysates were used in insect assays or mixed and then used, insecticidal activity was detected at a very low level. If the genes *xptB1* and *xptC1* were expressed in the same *E. coli* cell and this cell lysate was mixed with cells expressing *xptA1*, activity was restored to *P. rapae* and *P. brassicae*. Similarly mixing XptB1/C1 lysate with XptA2 lysate restored activity towards *H. virescens*. Individual gene disruptions in *X. nematophilus* PMFI296 reduced activity to insects; this activity was restored by complementation with cells expressing either *xptA1* or *xptA2* for their respective disruptions or *E. coli* expressing both *xptB1* and *xptC1* for individual disruptions of either of these genes. The genes *xptA2*, *xptC1*, and *xptB1* were expressed as an operon in PMFI296 and inactivation of *xptA2* or *xptC1* resulted in silencing of downstream gene(s), while *xptA1* was expressed as a single gene. Therefore, the two three gene product combinations interact with each other to produce good insecticidal activity.

New insecticidal toxins with activity towards pests of commercial importance are needed for either the development of sprayable products or transgenic plants. We have previously described a group of toxins from *Xenorhabdus* species that kill lepidopteran insects. The genes responsible for this insecticidal activity were identified by screening a cosmid genomic library, expressed in *Escherichia coli*. One cosmid (CHRIM1) contained five genes related to insecticidal activity (12). One of these genes (*xptA1*) was central for insecticidal activity towards *Pieris brassicae*, and when expressed in *E. coli*, a low level of insecticidal activity was observed. However for full insecticidal activity a much larger region of DNA comprising *xptA1* and at least two other genes, *xptB1* and *xptC1*, was required. The precise genetic nature of toxin combinations resulting in insecticidal activity and their effect upon other insects had not been determined.

Genes homologous to those present on CHRIM1 have also been described in *Photorhabdus luminescens* (1, 10), a symbiont of entomopathogenic nematodes closely related to *Xenorhabdus* species. The requirement of three genes equivalent to *xptA1*, *xptB1*, and *xptC1* in the expression of full insecticidal activity towards the model insect *Manduca sexta* (tobacco hornworm) was shown first in *P. luminescens* strain Hb (10) and later in *P. luminescens* strain W14 (13). Homologous genes to *xptA1*, *xptB1*, and *xptC1* have also been identified in *Serratia entomophila* (7), where some species are the causative agent of the chronic Amber disease in the New Zealand grass grub (8). Three plasmid-encoded genes, *sepA*, *sepB*, and *sepC*, homologous to *xptA1*, *xptB1*, and *xptC1*, were all required to be expressed in the same *E. coli* cell to induce full disease symptoms (7). In this previous work individual clones of each gene expressed from *E. coli* promoters could not be obtained due to rearrangements within the constructs. In this study we have expressed four *xpt* genes individually in *E. coli*: *xptA1* (*tcdA*/*sepA*-like; 7,841 bp; 287-kDa predicted protein), *xptA2* (*tcdA*/*sepA*-like; 7,647 bp; 285-kDa predicted protein), *xptB1* (*tccC*/*sepC*-like; 3,047 bp; 111-kDa predicted protein), and *xptC1* (*tcdB*/*sepC*-like, 4,256 bp; 160-kDa predicted protein). We have also determined which combinations of these genes need to be expressed for activity against different insect species. Gene disruptions in *Xenorhabdus* strains have also been produced to determine if similar interactions of the gene products occur in the wild-type strain. These aspects of *Xenorhabdus* insect toxins have not been addressed in earlier work. In addition the effect of the toxin complexes on four commercial pests have been studied in this work. With the potential of these genes for use in insect control, it is important that these points are addressed. In order to achieve this each of the *xpt* genes from PMFI296 were cloned individually into *E. coli* under the control of the λ P_L promoter, and cloned in combinations on the same vector, or on different vectors using the P_{LAC} promoter in the same cell. Cells and cell lysates were used individually or mixed prior to inclusion into insect bioassays.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The strain *Xenorhabdus nematophilus* PMFI296 was isolated from United Kingdom soil and has been described else-

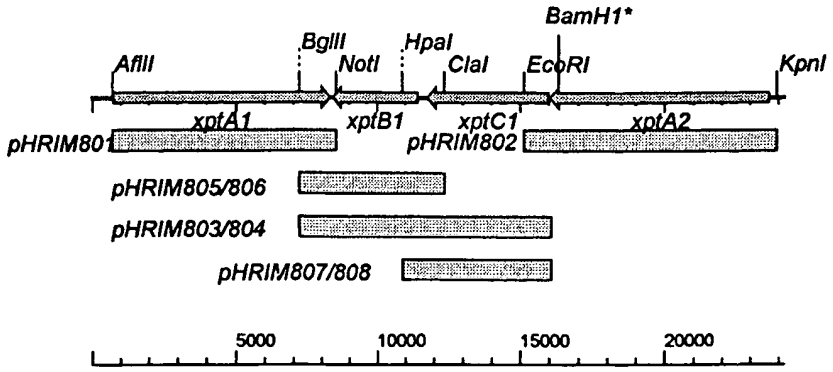

FIG. 1. Fragments of cHRIM1 which are present in plasmid constructs expressing *xpt* genes. The light-colored boxes show the regions of cHRIM1 which are present in each plasmid specified. Enzyme sites shown are those used in the subcloning (see text). *, *Bam*HI site that was introduced via an AT2 transposon. Sizes are indicated in base pairs.

where (12). The *E. coli* strain GI724 and the vector pLEX were obtained from Invitrogen (Groningen, The Netherlands). Regulation of gene expression on pLEX through the incorporation of tryptophan in the media has been described previously (12). Briefly, clones were maintained on RMG medium containing 100 μg of ampicillin ml^{-1} (Invitrogen), and expression was obtained by growth in LB broth (Merck, Darmstadt, Germany) containing 100 μg of ampicillin ml^{-1} and 100 μg of tryptophan ml^{-1}. pBBR1MCS (9) is a broad-host-range *inc*P plasmid that can be maintained in the same cell as plasmids that possess *col*E1-derived origins such as pLEX. The plasmid pBBR1MCS also contains a *lac* promoter upstream of the multiple cloning site that can be used for expression of cloned genes. Clones in pBBR1MCS were obtained on LB broth containing 50 μg of chloramphenicol ml^{-1}, and induction of the *lac* promoter was achieved by incorporating IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) into the growth medium.

DNA purification and subcloning. Plasmid DNA was prepared by the Qiagen (Dorking, United Kingdom) midi and Qiawell 8 (Qiagen) systems. Restriction digests were performed using the manufacturers' recommended conditions (Boehringer Mannheim, Lewes, United Kingdom; Life Technologies, Paisley, United Kingdom) and analyzed by agarose gel electrophoresis. After digestion, DNA was purified for cloning using the Qiagen PCR product clean-up system, following the manufacturer's recommended conditions (Qiagen). Blunt ending of DNA was performed using 1 U of Klenow (Life Technologies) in recommended buffer with 0.015 mM concentrations of deoxynucleoside triphosphates for 15 min at room temperature. The Klenow enzyme was inactivated by heating at 70°C for 10 min. All subcloning was carried out in *E. coli*, and DNA was electroporated into strains (12.5 $kVcm^2$) using a Bio-Rad GenePulser. Clones were selected on LB or RMG plates containing the appropriate antibiotics.

Cloning and expression of toxin genes. The construction of P_L-*xptA1* in plasmid pHRIM801 has been described elsewhere (12) and consists of the *xptA1* gene placed downstream of the λ P_L promoter in the *E. coli* strain GI724, where the cI element is present on the chromosome under the control of the *trp* operon (Invitrogen). The plasmid pHRI802 (P_L-*xptA2*) was created by cloning an 8,788-bp *Kpn*I/*Eco*RI fragment, containing the entire *xptA2* gene from cosmid CHRIM1, into pLEX cut with *Kpn*I/*Eco*RI. The plasmid pHRIM803 (P_{LAC}-B1/C1) was created from p338/2-AT2-191, an AT-2 transposon insertion mutation of clone 338/2 (12). The point of AT-2 insertion was identified as 171 bp upstream of the start codon of the *xptC1* gene in the following sequence GGA GAG CCT GAG CG*A TAT* CAT TCT GCA TAT CCG CT, such that ATATC was duplicated during transposition. A 9,708-bp fragment containing both the *xptB1* and *xptC1* genes was excised from p338/2-Tm191 with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of pMCS-BBRC-1 such that the *xptB1* and *xptC1* genes were in the correct orientation for expression from the *lac* promoter of pMCS-BBRC-1. Plasmid pHRIM804 (P_L-*xptB1/C1*) was similarly constructed by cloning the *Bam*HI/*Bgl*II fragment into the *Bam*HI site of pLEX. The plasmid pHRIM805 (P_L-*xptB1*) was created by cutting pHRIM804 with *Cla*I and *Bam*HI, treating with Klenow, and religating, thus removing a 4,521-bp fragment containing the majority of the *xptC1* gene. PHRIM806 (P_{LAC}-*xptB1*) was created by cutting pHRIM603 with *Cla*I and religating, thus again removing a 4,557-bp fragment containing the majority of the *xptC1* gene. The plasmid pHRIM807 (P_L-*xptC1*) was constructed by cloning an *Xho*I fragment from a pHRIM803 into pLEX. This construct was then cut with *Hpa*I/*Sma*I and religated, thus removing the majority of the *xptB1* gene. Plasmid pHRIM808 (P_{LAC}-*xptC1*) was constructed by removing the AT2 trimethoprim resistance cassette from pHRIM803 with a *Sal*I digest. This construct was cut with *Xba*I and *Hpa*I, blunt ending with Klenow and religated to form pHRIM808. Figure 1 summarizes the regions of DNA and genes present in each of the plasmid constructed.

Construction of a suicide plasmid for *X. nematophilus*. In order to insert the kanamycin resistance gene cassette into the *X. nematophilus* PMFI296 chromosome, an appropriate suicide vector was constructed using the *sacB* gene. The *sacB* gene codes for levan sucrase and is lethal in many gram-negative bacteria in the presence of 5% (wt/vol) sucrose (4). The gene was amplified from *Bacillus subtilis* 168 chromosomal DNA using the forward primer (5′-TAC CTG CAG AGT TCT TTA GGC CCG TAG-3′) and the reverse primer (TAC CTG CAG ATG CCA ATA GGA TAT CGG-3′), which incorporated *Pst*I restriction sites (underlined sequence) into the PCR product. Amplification was carried out as follows: 95°C for 30 s; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min; and a final extension of 72°C for 5 min. The amplified product was then cut with *Pst*I and cloned into the *Pst*I site of pBBR1MCS to form plasmid pHRIM810. Transformants containing pHRIM810 were selected on LB plated containing kanamycin (50 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}).

Gene disruptions in *Xenorhabdus*. The insecticidal genes of *X. nematophilus* PMFI296 (*xptA1*, *xptA2*, *xptB1*, and *xptC1*) were disrupted by insertion of a kanamycin resistance cassette (Pharmacia) into each gene. The first step in this process involved cloning the whole or part of the appropriate insecticidal gene into the multiple cloning site of the suicide vector pHRIM810 described previously. The kanamycin resistance cassette was then inserted into the insecticidal gene present on the suicide plasmid. Figure 2 shows the enzyme sites used for the kanamycin cassette insertion in each gene. The *E. coli* strain ED8654 harboring PNJ5000 (6) was then transformed with the appropriate gene disruption plasmid. Colonies were selected on LB agar containing kanamycin (50 μg ml^{-1}). Colonies were grown in LB medium containing kanamycin (50 μg ml^{-1}) at 37°C for 18 h

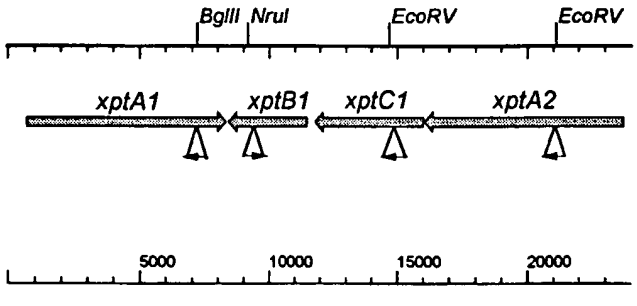

FIG. 2. Location of gene disruptions on the *X. nematophilus* PMFI296 chromosome. Arrows show the location and orientation of each kanamycin cassette. The enzyme sites shown are those that were used to introduce the cassette into each gene disruption plasmid. Sizes are indicated in base pairs.

and diluted 1/100 into fresh medium. The cultures were incubated at 37°C until an optical density at 600 nm of 0.5 was reached. A 25-ml aliquot was centrifuged (4,000 × *g* for 10 min at 15°C), and the pellet was washed in 25 ml of LB. The recipient strain was prepared for mating in a similar way. *X. nematophilus* PMFI296 was grown in 5 ml of LB at 30°C overnight. Cells were collected and washed with 25 ml of LB by centrifugation (4,000 × *g* for 10 min at 15°C). Both cell pellets were resuspended in 0.2 ml of LB. A subsample, 0.1 ml of each, was mixed and pipetted onto 1.4-cm diameter, 0.2-μm-pore-size nitrocellulose filters that had been placed on LB plates. Samples were incubated for 16 h at 30°C. Growth on the filter was scraped into 1 ml of LB and vortexed to resuspend the cells. Transconjugates were selected by plating these cells onto LB agar containing 100 μg of ampicillin ml^{-1} and 50 μg of kanamycin ml^{-1}, taking advantage of *Xenorhabdus* species natural resistance to ampicillin. The *X. nematophilus* PMFI296 strains containing each gene disruption plasmid were grown in 5 ml of LB containing 25 μg of kanamycin ml^{-1} for 16 h at 30°C, and dilutions were plated on LB agar containing 25 μg of kanamycin ml^{-1} and 5% (wt/vol) sucrose. After incubation at 30°C for 48 h, colonies that grew through the sucrose selection were screened for plasmid loss by testing for chloramphenicol resistance. Colonies that were chloramphenicol sensitive were characterized by Southern blot analysis to confirm insertional inactivation within the correct gene. For the Southern blot, DNA was obtained from the strains using Qiagen chromosomal DNA kit, restricted with *Eco*RI and *Hin*dIII, and probed with digoxigenin-labeled PCR products approximately 500 bp in length that corresponded to the inactivated gene. The probes were chosen such that different size fragments would be highlighted for *Hin*dIII- and *Eco*RI-restricted DNA from the wild type, the insertion mutant, and the suicide plasmid. In each case the Southern blot revealed a single band corresponding to the correct predicted size for the insertion mutant (data not shown).

Insecticidal assays. Initial bioassays to assess activity of *E. coli* clones against *P. brassicae* involved growing the strains in 50 ml of LB for 16 h. Cells were harvested by centrifugation of 1 ml of culture (13,000 × *g* for 2 min), the supernatant was removed, and the pellet was resuspended in 1 ml of PBS (10 mM phosphate buffer, pH 7.4; 2.7 mM KCl; 137 mM NaCl). The cells were lysed by sonication at 18 Ω for 20 s, and the potency of the lysate or a mixture of lysates was tested in incorporation assays. These were performed by spreading 50 μl of lysate onto agar based artificial diet (3) which contained streptomycin (20 μg ml^{-1}) and cefotaxime and tetracycline (each at 100 μg ml^{-1}) in plastic containers (diameter, 4.5 cm). After the surface had dried, 10 larvae were added and containers were incubated at 25°C (16-h day-length period) and relative humidity of 80%. Recordings of larval mortality were taken after 24 h. A positive result was scored if all larvae were dead, and a negative result if no larvae had died. In the assays negative controls included treatments with just buffer (PBS) and *E. coli* cells containing pLEX. More detailed bioassays to study the activity of *X. nematophilus* PMFI296, disruption mutants, and *E. coli* clones against neonate *P. brassicae*, *Heliothis virescens*, and *Plutella xylostella*, as well as *E. coli* clones against *Pieris rapae* larvae were performed as follows. Cell samples of *X. nematophilus* PMFI296 and the disruption mutants were each prepared from cultures grown for 72 h at 25°C on eight 9-cm-diameter petri plates containing L agar. Cells were harvested, suspended in 200 ml of PBS, washed by centrifugation at 6,000 × *g* for 10 min, and resuspended in 15 ml of 5% (wt/vol) lactose. Cell suspensions were then frozen at 70°C for 4 h and freeze-dried at −60°C for 48 h. For *E. coli*, cells were cultured in 200 ml of LB containing 50 μg of ampicillin ml^{-1} for 40 h at 30°C. Cells were harvested by centrifugation, washed once in PBS, and suspended in 8 ml of PBS. The cells were then lysed by sonication using three bursts of 20 s at 18 Ω. To the cell lysate, 8 ml of 10% lactose (wt/vol) was added, and the resulting cell suspension was frozen and freeze-dried as described above. To measure the amount of total protein present in the freeze-dried samples the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) was used, and the manufacturer's instructions were followed. The amount of XptA1, XptA2, and XptC1 in the material was calculated by analyzing the samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and calculating the relative intensity of the appropriate toxin band to the sum of all the other bands using Phoretix 1D Analysis Software (version 4.01; BioGene). The same freeze-dried samples were used in all experiments to ensure the amount of toxin protein in the samples remained constant. To determine the potency of the bacterial samples each was tested in triplicate using multidose assays on artificial diet. These were performed using a series of five dilutions for each sample. For each dilution, 50-μl duplicates of bacterial suspension was tested against *P. brassicae*, *P. rapae*, and *P. xylostella* in spread assays as already explained. For *H. virescens*, four containers per dilution were used, to which five larvae were added. Recordings of *P. brassicae*, *P. rapae*, and *P. xylostella* larval mortality were taken after 6 days. For *H. virescens*, activity was measured as reduction in larval weight compared to that of untreated controls after 5 days of growth at 25°C. In all the assays, negative controls included treatments with just PBS. The results of the assays were evaluated by Logit transformation using Genstat (5th edition; VSN International Ltd.) to determine the 50% lethal concentration (LC_{50}) and the concentration required to cause a 50% reduction in larval weight compared to an untreated control (EC_{50}).

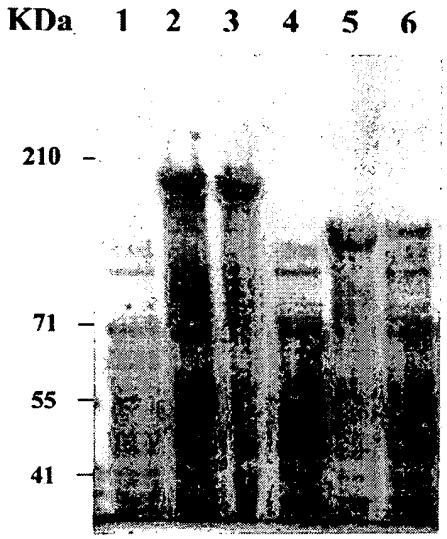

FIG. 3. SDS-PAGE of cell lysates from *E. coli* clones expressing *xpt* genes. Lanes: 1, GI724(P_L); 2, GI724(P_L-*xptA1*); 3, GI724(P_L-*xptA2*); 4, GI724(P_L-*xptB1*) 5, GI724(P_L-*xptC1*); 6, GI724(P_L-*xptB1/C1*). Markers are indicated at left.

SDS-PAGE of cell proteins. *E. coli* clones were grown in induction media for 16 h and cells were harvested by centrifugation at 13,000 × *g* for 5 min. The cell pellet was resuspended in one-eighth of the original volume of PBS. Samples (5 μl) were added to equal volumes of 2× SDS-PAGE loading buffer (100 mM Tris HCl, pH 6.8; 1% mercaptoethanol; 4% SDS; 20% glycerol; 0.2% bromophenol blue) before being placed in a boiling water bath for 5 min. Samples were then loaded onto 3 to 8% precast gradient gels (Novex, San Diego, Calif.), which were run at 150 V for 1.5 h. Gels were stained with 0.25% (wt/vol) Coomassie brilliant blue in 40% (vol/vol) methanol–10% (vol/vol) acetic acid for 1 h and destained for 3 h.

RESULTS

Expression of genes. Four genes present on the CHRIM1 cosmid, *xptA1*, *xptA2*, *xptB1* and *xptC1* were cloned into expression vectors. When gene expression was induced, the constructs P_L-*xptA1*, P_L-*xptA2* and P_L-*xptC1* were expressed at high levels as judged by distinct bands on Coomassie blue stained SDS-polyacrylamide gels (Fig. 3). Each band was N-terminally sequenced and in each case the first 10 amino acids matched exactly with the predicted open reading frames (ORFs) of *xptA1*, *xptA2*, and *xptC1* genes, respectively. The predicted size of *xptB1* is 110 kDa, but no protein of this size (or any other size) could be detected when *xptB1* was expressed alone or in the presence *xptC1* (lanes 4 and 6). Although the XptC1 protein band visible in P_L-*xptB1/C1* constructs (lane 6) was less intense than when *xptC1* was expressed alone, it was confirmed to be *xptC1* by N-terminal sequencing of the first 10 amino acids. The predicted XptB1 protein contains repeating peptide motifs termed YD repeats (14), which in certain proteins have been shown to bind to high molecular mass carbohydrates preventing the protein from being detected by SDS-PAGE (11). To see whether this was the case with *xptB1*, *E. coli*

TABLE 1. Effect on *P. brassicae* of mixing lysates from strains of *E. coli* expressing different *xpt* genes

Strain 1	Strain 2	Activity of mixture[a]
GI724(P_L-*xptA1* + P_{LAC}-*xptB1/C1*)	GI724(P_L)	+
GI724(P_L-*xptA1*)	GI724(P_{LAC}-*xptB1/C1*)	+
GI724(P_L-*xptA1*)	GI724(P_L-*xptC1* + P_{LAC}-*xptC1*)	+
GI724(P_L-*xptA1*)	GI724(P_L-*xptC1* + P_{LAC}-*xptB1*)	+
GI724(P_L-*xptA1* + P_{LAC}-*xptB1*)	GI724(P_L-*xptC1*)	−
GI724(P_L-*xptA1* + P_{LAC}-*xptC1*)	GI724(P_L-*xptB1*)	−
GI724(P_L-*xptA1*)	GI724(P_L)	−
GI724(P_L-*xptB1/C1*)	GI724(P_L)	−

[a] Symbols: +, all larvae were dead after 48 h; −, no larval mortality after 48 h.

cells expressing *xptB1* alone and in conjunction with *xptC1* were sonicated directly in 8 M urea and loaded onto SDS-polyacrylamide gels, but still no protein could be detected. However, when analyzing lysates from *E. coli* containing P_{LAC}-*xptB1/C1* or P_L-*xptB1/C1* constructs using Mono-Q ion-exchange fast-performance liquid chromatography (Pharmacia), fractions where the visible XptC1 protein dominated contained a second prominent protein of approximately 65 kDa, whose N-terminal sequence (10 amino acids) matched that of the predicted XptB1 protein. Therefore, a truncated XptB1 protein is produced in these constructs which is difficult to detect. The P_{LAC} clones showed expression patterns similar to those of the P_L constructs when examined by SDS-PAGE, with both GI724(P_{LAC}-*xptC1*) and GI724(P_{LAC}-*xptB1/C1*) expressing XptC1, albeit at about 10-fold-lower levels due to the weaker P_{LAC} promoter. As with the P_L constructs, no XptB1 product could be detected by SDS-PAGE.

Interaction of gene products for insecticidal activity. Initial studies involved elucidating which genes were involved in activity against *P. brassicae* (Table 1). The *E. coli* strain GI724 (P_L-*xptA1* + P_{LAC}-*xptB1/C1*) was shown to exhibit insecticidal activity toward *P. brassicae* (all larvae were dead after 48 h using undiluted lysate from overnight cultures), but individually P_L-*xptA1* and P_L-*xptB1/C1* showed no activity (no larvae dead after 48 h). Thus, interaction between the *xptA1* and *xptB1-xptC1* genes was essential for activity. This interaction need not occur in vivo as exhibited by the toxic activity of mixed lysates of P_L-*xptA1* and P_L-*xptB1/C1*. In addition, mixing of the lysates from P_L-*xptA1* and either P_L-*xptC1* + P_{LAC}-*xptB1* or P_L-*xptB1* + P_{LAC}-*xptC1* also resulted in insecticidal activity. Therefore the *xptC1* and *xptB1* genes do not need to be collinear in order to interact. However, both genes do need to be expressed in the same cell, as shown by lack of insecticidal activity when *xptB1* and *xptC1* were expressed in separate cells (Table 1).

Effect of gene products on different insect species. Once the correct combination of genes required for toxicity had been elucidated more detailed studies on a range of Lepidoptera were performed. The effects of GI724(P_L-*xptA1*) and GI724 (P_L-*xptA2*) alone and in conjunction with P_L-*xptB1/C1* against different species of insect are shown in Table 2. The data show that *E. coli* strains expressing *xptA1* when mixed with strains expressing *xptB1* and *xptC1* showed activity towards *P. brassicae* and *P. rapae*, whereas *xptA2* in conjunction with *xptB1* and *xptC1* showed significant activity toward *H. virescens*. These results clearly illustrate that the *xptA1* and *xptA2* genes exhibit a different spectrum of activity, but both require the *xptB1* and *xptC1* gene products in order to produce full insect activity.

Activity of strains with gene disruptions. Table 3 shows the results of insecticidal assays involving strains of *X. nematophilus* PMFI296 with gene disruptions. A mutant with the *xptA1* gene disrupted by a kanamycin cassette, PMFI296 (*xptA1*::kan), showed 10% of the activity toward *P. brassicae* than the PMFI296 wild type, but activity could be restored by adding XptA1 expressed in *E. coli*. This confirmed that XptA1 protein is essential for activity towards this pest. PMFI296 (*xptB1*::kan) and PMFI296 (*xptC1*::kan) strains also exhibited substantially less activity than the wild type to *P. brassicae*, but activity could not be restored by adding lysate from *E. coli* expressing either *xptB1* or *xptC1* singly. PMFI296 (*xptB1*::kan) and PMFI296 (*xptC1*::kan) strains could be complemented with *E. coli* strains expressing both *xptC1* and *xptB1* together. These results supported the earlier findings that the *xptB1* and *xptC1* genes need to be in the same *E. coli* cell in order to be able to complement *xptA1*. Similarly both genes have to be expressed within the same cell in *Xenorhabdus* strains.

The results from PMFI296 (*xptA2*::kan, *xptC1*::kan, or *xptB1*::kan) mutants confirmed, as expected, that the contiguous genes *xptA2*, *xptC1* and *xptB1* were expressed as a single polycistronic operon within the wild-type strain. The kanamy-

TABLE 2. Activity of lysates from single *xpt* genes and combinations of cloned *xpt* genes in *E. coli* GI724

Gene(s)	LC_{50}/ng of toxin for:			ED_{50}/ng of toxin for *H. virescens*
	P. brassicae	*P. rapae*	*P. xylostella*	
xptA1[c]	1,170 (740–1,850)[b]	>5,000	>5,000	>2,000
xptA2[c]	>5,000	>5,000	>5,000	>2,000
xptB/C[c]	>5,000	>5,000	>5,000	>2,000
xptA1 + *xptB/C*[d]	3.20 (2.13–5.76)	45.1 (31.5–64.4)	>5,000[a]	>2,000
xptA2 + *xptB/C*[d]	306 (195–481)	>5,000	>5,000	5.25 (3.85–7.00)
xptA1 + *xptA2* + *xptB/C*[d]	5.85 (3.90–9.36)	93.5 (65.5–133)	>5,000[a]	6.63 (4.68–8.97)

[a] Some reduction in size.

[b] 95% confidence limits are given in parentheses.

[c] Data reported as LC_{50} or ED_{50} per ng of toxin (XptA1, XptA2, or XptC1) present in the lysate. The amount of XptA1, XptA2, and XptC1 in each lysate as a percentage of total protein was protein was 4.7, 4.6, and 2.8, respectively. XptB1 was below detectable levels, and therefore its concentration was not used in the calculations.

[d] Data reported as LC_{50} or ED_{50} per ng of combined toxins present in the lysate. The ratio of XptA2/A1 to XptC1 in the mixed lysate was approximately 1.6:1, and that for XptA1 to XptA2 to XptC1 was approximately 1.6:1.6:1.

TABLE 3. The activity of *X. nematophilus* gene knockout mutants and cloned toxin genes found to supplement insect activity

Strain or mutant	LC_{50} (μg of protein per cm^2 of diet) vs:			ED_{50} (μg of protein per cm^2 of diet) vs:	
	P. brassicae	*P. brassicae* + suppl.[a]	*P. xylostella*	*H. virescens*	*H. virescens* + suppl.[a]
PMF1296	0.04 (0.02–0.05)[b]		0.96 (0.62–1.38)	0.07 (0.06–0.09)	
A1::Kan	0.54 (0.39–0.83)	0.06 (0.05–0.09) [A1]	2.14 (1.43–2.90)	0.06 (0.04–0.09)	
A2::Kan	0.65 (0.49–0.87)	0.04 (0.29–0.06) [B/C]	31.03 (23.8–39.5)	>50	0.57 (0.42–0.81) [A2+B/C]
C1::Kan	1.65 (1.27–1.93)	0.13 (0.09–0.17) [B/C]	51.12 (39.2–69.7)	>50	0.32 (0.21–0.52) [B/C]
B1::Kan	2.05 (1.64–2.54)	0.15 (0.13–0.19) [B/C]	69.04 (53.9–81.6)	>50	0.33 (0.19–0.54) [B/C]

[a] Knockouts mutants of *X. nematophilus* mixed with extracts of *E. coli* containing cloned toxin genes (in brackets), found to supplement insect activity. A1, XptA1; A2, XptA2; B/C, XptB1/C1.
[b] Values in parentheses are 95% confidence limits.

cin cassette was constructed with a transcriptional terminator, which when inserted into a gene would result in truncation of a polycistronic transcript. In this approach it would silence transcription of downstream genes. As such, an *xptA2* insertion would silence *xptC1* and *xptB1*, while an *xptC1* insertion would silence *xptB1*. PMFI296 (*xptA2*::kan) mutants showed reduced activity toward *P. brassicae*, but results obtained from *E. coli* expressing *xptA2* (Table 2) showed this gene was not involved in activity toward this insect. The addition of XptB1/C1, but not XptA2 to PMFI (*xptA2*::kan) strains restored activity against *P. brassicae* to wild-type levels. These results confirmed that *xptA2*, *xptC1* and *xptB1* were expressed as a polycistronic transcript.

PMFI (*xptA1*::kan) mutants showed activity comparable to the wild type against *H. virescens*, confirming that this gene was not involved in activity towards this insect, and that lack of expression of XptA1 did not change expression or activity of *xptA2*, *xptC1*, and *xptB1*. However *xptA2* and *xptB1xptC1* disruptions reduced significantly the activity towards *H. virescens*. Addition of XptA2 to PMFI296(*xptA2*::kan) strains did not restore insecticidal activity to *H. virescens* confirming previous results that *xptC1* and *xptB1* were also silenced. Insecticidal activity for strains with *xptC1* and *xptB1* gene disruptions could be complemented with XptC1/XptB1 produced in the same *E. coli* strain. However, insecticidal activity could not be restored by adding lysate from *E. coli* expressing singly either *xptB1* or *xptC1*, for their respective gene disruptions. These results follow the same pattern that was observed for activity of PMFI *xpt* disruption mutants against *P. brassicae*.

DISCUSSION

This study shows that for full insecticidal activity, as for homologous genes in *S. entomophila* and *P. luminescens*, interactions between the products of three *xpt* genes (*xptA*, *xptB*, and *xptC*) are required. Also, for the first time, *xptA*- and *xptC*-like genes have been individually expressed in *E. coli* and an *xptB1*-like protein fragment has been detected. Previous attempts to express *xptA*-like genes from *P. luminescens* in *E. coli* have proved unsuccessful. Plasmids containing only the *tcdB* gene from *P. luminescens* could not be constructed due to rearrangement (13) and plasmids containing the *sepA* from *S. entomophila* were unstable in *E. coli*, suggesting that the SepA protein is detrimental to the growth of the host bacterium (7). The successful cloning of *xptA1* and *xptA2* in these studies may be due to different properties of these genes compared to *sepA* and *tcdB*, or more likely it is due to the tight regulation of their expression in the pLEX constructs. If the XptA1 and XptA2 proteins are deleterious to the host cell, then the fact that they are not expressed under regulated growth conditions in the pLEX system, would remove the pressure for rearrangements.

Also, importantly, the effect of these genes on two commercial pests, *P. rapae* and *H. virescens*, has been elucidated, and it has been found that different spectrums of activity can be achieved by substituting different *xptA* genes with the same *xptB1-xptC1* construct. This is important because to date, the effect of the toxin genes of *Photorhabdus* and *Serratia* have only been studied in detail on the model insect *M. sexta* and on *Costelytra zealandica*, which is a pest of New Zealand grasslands. Further adding to our understanding of *xpt* like genes are the findings that the interaction between XptA1 and XptB1/C1 can occur in vitro by mixing cell lysate, but the interaction of the *xptB1* and *xptC1* genes requires their expression in the same bacterial cell.

Interestingly, disruption of the *xptC1*, *xptB1*, or *xptA2* genes reduced the activity of PMFI296 mutants against *P. xylostella* by more than 30-fold (Table 3). However, when lysate from an *E. coli* expressing *xptA2* was mixed with a lysate of *E. coli* expressing *xptB1* and *xptC1* no activity towards *P. xylostella* was observed (Table 2). Therefore, there is possibly another unidentified insecticidal toxin gene in PMFI296, which requires XptB1/XptC1 proteins for activity and is responsible for activity towards *P. xylostella*. Alternatively the *xptA2* gene may be responsible for such activity but is inactive in *E. coli* due to lower levels of expression or other factors present in *Xenorhabdus* but lacking in *E. coli*. The latter is more likely since *xptA2* is highly expressed in P_L-*xptA2* constructs.

Previous work on XptA-like toxins in *P. luminescens* showed that single purified XptA1 like proteins were sufficient for toxicity (5), but when the genes coding for these proteins were expressed in *E. coli*, insect activity was not conferred (1). In light of the present work and other research (13), one explanation for this could be that tiny undetectable amounts of XptC1 and XptB1 like proteins may have contaminated the protein preparation in these protein studies, and contributed to the toxicity observed. Alternatively, processing of the XptA1 protein by XptC1 and XptB1 like proteins may have occurred in the *Photorhabdus* cell before purification. Therefore, the proteins purified represent the processed or active forms of XptA1.

From the results presented here, processing of an inactive XptA type protein by the XptB1/C1 complex to produce an active XptA protein remains a possible theory. The fact that

XptA1 shows slight toxic effects on its own, alongside the fact that purified homologues from *P. luminescens* W-14 were active supports this theory. The different spectrum of activities exhibited by XptA1 and XptA2 is also consistent with this theory. If this is the case then it seems likely that the XptB1/C1 complex is capable of activating a range of XptA like molecules with differing spectrums of activity. Such activation of any XptA1 like proteins may not be easy to detect because SDS-PAGE and matrix-assisted laser desorption-ionization time-of-flight analysis showed that the size of the active purified TcaA and TcaB proteins were equal to the size predicted by their ORFs (5). In our experiments no differences in the protein pattern of XptA1 was observed when expressed alone, or in the presence of XptB1/C1. Therefore, protease activity or any covalent modification by the XptB1/C1 complex that increases or decreases the size of the XptA1 protein significantly, is unlikely.

Coexpression of *tcdA* (*xptA*-like) and *tcdB* (*xptC*-like) in *E. coli* resulted in formation of a phage-like structure, visible in toxic particulate preparations. Expressing *tccC* (*xptB*-like) in the same cell as *tcdA* and *tcdB* does not alter these structures but renders them orally toxic (13). We have looked at crude lysates from recombinant *E. coli* and have been unable to observe these structures. However, our expression constructs, unlike those used for expressing *tcdA* and *tcdB*, do not contain phage-like genes such as *lysR*. In addition, our cultures are also not induced with UV, which may result in expression of endogenous prophage proteins. Both of these factors may aid in the formation of phage-like structures seen with recombinant *tcdA* and *tcdB*. If formation of these phage-like structures is essential for toxicity, then such formation would have to take place outside the cell since *xptA1/xptA2* and *xptB1/C1* lysates can be mixed in vitro to produce insect activity.

In this study the interactions seen with *E. coli* expressed proteins was reflected in those expressed in the wild type *Xenorhabdus* strain. This moves the interactions recorded away from transcriptional and translational interactions, to posttranscriptional modifications or protein interactions. These interactions are not additive. This study showed that *xptA2*, *xptC1*, and *xptB1* were expressed as a single polycistronic message, and *xptA1* was expressed independently. Disruptions in *xptA2* using a trimethoprim gene cassette with no transcriptional terminator allowed the expression of the downstream genes *xptC1* and *xptB1*, and maintenance of activity against *P. brassicae* (12). In this study, disruption of *xptA2* with a kanamycin resistance gene cassette with a transcriptional terminator, prevented downstream expression of *xptC1* and *xptB1* and loss of insecticidal activity. Therefore in *Xenorhabdus*, the genes *xptA2*, *xptB1*, and *xptC1* are colocalized and are expressed on a polycistronic message from promoters that also function in *E. coli*. In *P. luminescens* W14, the genes *tcb*, *tcd*, *tca*, and *tca* are clearly different in this regard, since they are distributed separately over the chromosome, and expressed individually from inducible phage-like promoters (2). They are also expressed in an uncoordinated fashion at different times during growth. In *Serratia* although the *sep* genes are more tightly grouped, an ORF still separates *sepB* and *sepC* and there is no *xptA1*-like gene equivalent in the arrangement. Thus, the arrangement of these genes in *Xenorhabdus* may represent a more evolved structure, where over time distal mobile genes which are able to interact for a beneficial effect, i.e., insect toxicity, have, through numerous integration events, formed a tightly integrated unit.

Clearly questions remain relating to how *xpt* gene products interact to form an active toxin. Since XptA1 and XptA2 individually and XptB1 and XptC1 combined show a low level of insecticidal activity, they may all be active toxins. In this way the interaction of the toxins, either before they come into contact with the target cell, or after this interaction, could result in an effect which kill insects quicker. However, the fact that we now have individual proteins expressed at high levels in *E. coli* and some combinations are active when mixed in vitro, this will help answer some of these questions. Further studies on purified proteins expressed in *E. coli* and their interactions are needed.

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